New derivatives of 4,5,6,7-tetrabromobenzimidazole and method of their preparation

FIELD OF THE INVENTION

The object of the invention are new derivatives of 4,5,6,7-tetrabromobenzimidazole and a method of their preparation.

BACKGROUND OF THE INVENTION

An unsubstituted 2-amino-4,5,6,7-tetrabromobenzimidazole found in fruits as a product of degradation of the anthelmintic agent Benomyl (Pease and Gardiner, 1969) is known in the art. Its structure was proposed on a basis of mass spectrum, however its synthesis has not been reported yet.

From US Patent Application No. 2003/0027842A1, 2-hydroxy-4,5,6,7-tetrabromo-benzimidazole, obtained by bromination of 3–carboxy-4,6,7-tribromo-2-hydroxybenzimidazole, and 1-alkyl-4,5,6,7-tetrabromobenzimidazole, prepared by alkylation of 4,5,6,7-tetrabromobenzimidazole with alkyl halide in alkaline solution, are known.

Halogeno derivatives of benzimidazole exhibit many interesting biological properties. It is known that derivatives of 2-trifluorobenzimidazole as well as some derivatives of bromobenzimidazoles have marked antiprotozoic, antibacterial and antiviral activity (Navarete-Vazquez et al. Bioorg.Med.Chem.Lett.11, (2001), 187-191[1]; Andrzejewska et al. Eur.J.Med.Chem. 37 (2002), 972-978)[2]. The most probable explanation for considerable biological activity of halogeno derivatives of benzimidazole is their ability to interfere with cell metabolism by inhibition of enzymes that control metabolism of protein kinases.

Protein kinases constitute a large superfamily of enzymes (more than 500 members encoded by the human genom) present in every eukaryotic and prokaryotic cell and playing a special role of regulators of cellular mechanisms. The enzymes catalyse transfer of phosphate group from ATP or GTP to amino acids i.e. serine, threonine or tyrosine. These are in general „quiet" enzymes, which however activate themselves at individual stages, including also pathological stages, of cell metabolism. At present, protein kinases and particularly their inhibitors arouse the interest of researchers as potential objects that would help in design of specific drugs. For example, Gleevec, a drug to treat chronic myeloid leukemia (Druker et al. 2001)[3] is a result of studies on kinase inhibitors.

A special family of protein kinases are so-called casein kinases (CK1 and CK2), for which more than three hundred proteins - substrates for these enzymes - exist. They participate in many cell processes (Litchfield, 2003)[4]. There are many facts indicating that their subunits can act as

oncogenes (Kelliher et al., 1996, Orlandini et al, 1998)[5,6,7]. The most widely known and potent inhibitor of casein kinase 2 (CK2) is 4,5,6,7-tetrabromobenzotriazole (TBB)(Sarno et al., 2001).

Pharmacological study:

A cytotoxic effect of 2-dimethylamino-4,5,6,7-tetrabromo-benzimidazole on human leukemia Jurkat T cells was studied.

1. Leukemia Jurkat cells were incubated for 24 hours in a medium of increasing concentration of 4,5,6,7-tetrabromobenzimidazole (TBB) or 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole (K25). The cytotoxic effect was assessed by means of staining with a use of MTT {3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltriazolium bromide} reagent. Control cells were incubated in a medium with added solvent (DMSO, 05% v/v). The results were calculated as mean values of three experiments. The resulting cytotoxic effect of 4,5,6,7-tetrabromo-2-dimethylaminobenzimidazole on human leukemia Jurkat T cells is presented on Fig. 1. The results confirm that 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole is several times more active than tetrabromo-benzotriazole – a well-known inhibitor of casein kinase 2.

2. Leukemia Jurkat cells were incubated for 14 hours without addition of 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole (C), and in a medium including 5 or 10 uM of 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole and 50 ng/mL of antiFas (aF), a control substance to follow apoptosis process. Activation of caspase – an enzymatic protein controlling apoptosis was found while observing degradation of two proteins - substrates of that enzyme - HS1 and PARP. The effect is presented on Fig 2. The results indicate that molecular mechanism of action of 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole can be elucidated by induction of apoptosis -programmed cell death.

Tests of enzymatic activity of kinases were carried out using methodology described by Sarno et al. 2003 and are summarized in Table 1. The residual activity was determined by incubation in the presence of inhibitor at concentration 10μM and expressed as percentage activity without an inhibitor.

Table 1. Tests of enzymatic activity of kinases.

No.	Kinase*	4,5,6,7-Tetrabromo-benzotriazole (TBB)	2-Dimethylamino-4,5,6,7-tetrabromobenzimidazole (K25)
1.	CK1	91	87
2.	**CK2**	13	4
3.	G-CK	95	92
4.	**DYRK1a**	22	2

*CK1 – casein kinase 1, CK2 – casein kinase 2, G-CK- casein kinase from Golgi apparatus, DYRK1a – (Dual-specificity tyrosine phosphorylated and regulated kinase) Tyrosine kinase having regulating properties.

Experimental section

Materials

Native CK1 (nCK1) and CK2 (nCK2) were purified from rat liver (Meggio et al., 1981); Golgi CK (G-CK), purified from rat lactating mammary gland, was provided by Dr. A.M. Brunati (Padova, Italy). Protein tyrosine kinases Lyn, c-Fgr, Syk (also termed TPK-IIB) were purified from rat spleen as previously referenced (Sarno et al., 2003). Human recombinant α and β subunits of CK2 were expressed in E. coli and the holoenzyme was reconstituted and purified as previously described (Sarno et al., 1996). The V66A and I174A CK2 mutants were obtained as described previously (Sarno et al., 2003). V66AI174A double mutant was obtained with the "QuikChange-SiteDirected Mutagenesis" kit (Stratagene), using human V66A cDNA α inserted in pT7-7 vector as template and two synthetic oligonucleotide primers, 5'-GCACAGAAAGCTACGACTAGCAGACTGGGGTTTGGC-3' and 5'-GCCAAACCCCAGTCTGCTAGTCGTAGCTTTCTGTGC-3', each complementary to opposite strands of template. Expression and purification of the mutant was performed as previously described (Sarno et al., 2003).

Saccharomyces cerevisiae piD261 was provided by Dr. S. Facchin (Padova, Italy). The source of all the other protein kinases used for specificity assays is either described or referenced by Davies et al. (2000).

Kinetic determination:

Initial velocities were determined at each of the substrate concentration tested. Km values were calculated either in the absence or in the presence of increasing concentrations of inhibitor, from Lineweaver-Burk double-reciprocal plots of the data. Inhibition constants were then calculated by linear regression analysis of Km/Vmax versus inhibitor concentration plots. Considering that all TBI derivatives behave as competitive inhibitors with respect of ATP, inhibition constants were also deduced from the IC_{50}/K_i Cheng-Prusoff relationship (Cheng and Prusoff, 1973) by determining IC_{50} for each compound at 1 μM ATP concentration.

CK2 phosphorylation assays:

Phosphorylation assays were carried out in the presence of increasing amounts of each inhibitor tested in a final volume of 25 μl containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, 100 μM synthetic peptide phosphorylation substrate - RRRADDSDDDDD and 0.02 μM γ-^{33}P-ATP (500-1000 cpm/pmole), unless otherwise indicated, and incubated for 10 minutes at 37 °C. Assays were stopped by addition of 5 μl of 0.5 M orthophosphoric acid before spotting aliquots onto phosphocellulose filters. Filters were washed in 75 mM phosphoric acid (5-10 ml/each) four times then once in methanol and dried before counting.

Cell culture, treatment, and viability assay:

The human leukemia Jurkat T-cell line was maintained in RPMI-1640, supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. For the treatment, cells were suspended at a density of 10^6 cells/ml in a medium containing 1% (v/v) foetal calf serum, then incubated at 37 C, in the presence of the compounds at the indicated concentrations. Control cells were treated with equal amounts of solvent inhibitor was solved. At the end of incubations, cells were lysed by the addition of hypoosmotic buffer, as previously described (Sarno et al., 2003).

Cell viability was assessed by means of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltriazolium bromide (MTT) reagent, while caspase activation was followed by Western blot monitoring of PARP and HS1 protein degradation, as previously described (Ruzzene et al., 2002).

Surprisingly, experimental data obtained as a result of the present work indicate that the synthesized new compounds being the subject of the invention, while retaining the benzene moiety brominated at four positions, which is well fit into the hydrophobic excavation in the vicinity of ATP binding site, and modifying triazole fragment of the molecule, exhibit much better properties when compared to the known 4,5,6,7-tetrabromobenzotriazole (TBB) – one of the most effective and selective inhibitors of casein kinase 2.

It has been found that replacement of nitrogen atom at position 2 by carbon atom makes it possible to perform further modifications in that position more easily by replacement of hydrogen atom at position 2 with hydrophilic groups, which, in turn, makes it possible to establish new van der Waals interactions or form hydrogen bonding with polar kinase chains.

It appeared that among synthesized inhibitors - benzimidazole derivatives, the most effective is 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole (K25), for which inhibition constant (40nM) is the lowest among values observed up to now for CK2 inhibitors and is one order lower than that for TBB. It should be pointed out that K25 selectivity, comparable with TBB, because only one kinase, i.e. DYRK1a among more than 30 kinases examined, exhibits similar susceptibility to inhibition by K25. The more important is however cytotoxicity of K25 which is several times higher when compared to TBB in relation to leukemia Jurkat cells. K25 unlike TBB does not cause depolarization of isolated mitochondria. All that indicates undoubtedly that K25 has greater advantages than TBB, especially in case of *in vivo* studies.

We have found experimentally that some 2-amino-4,5,6,7-tetrabromobenzimidazoles substituted on the amino group, are more potent inhibitors CK2 than TBB. The respective results are summarized in Table 2. Thus, the above-mentioned 2-amino-4,5,6,7-tetrabromobenzimidazoles

substituted on the amino group can be useful as potential drugs as well as reagents for tests in molecular biology.

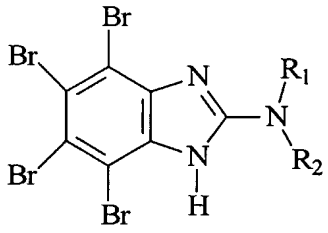
Table 2. Efficacy of 2-amino-4,5,6,7-pentabromobenzimidazole derivatives as CK2 inhibitors.

No.	Compound	Inhibition constant K_i (μM)
1.	4,5,6,7-tetrabromobenzotriazole (TBB)	0.30
2.	2-amino-4,5,6,7-tetrabromobenzimidazole	0.09
3.	2-methylamino-4,5,6,7-tetrabromobenzimidazole	0.09
4.	2-dimethylamino-4,5,6,7-tetrabromobenzimidazole (K25)	0.04
5.	2-etanoloamino-4,5,6,7-tetrabromobenzimidazol	0.13
6.	2-isopropylamino-4,5,6,7-tetrabromobenzimidazole	0.06
7.	2-(2-hydroxy)propyloamino-4,5,6,7-tetrabromobenzimidazole	0.14
8.	2-dimethylaminoethylamino-4,5,6,7-tetrabromobenzimidazole	0.16

Considering the state of the art that indicates high expression of casein kinase in the brain of patients suffering from Down's syndrome as well as in case of Alzheimer's disease, in which overproduction of kinases DYRK1a takes place, it seems very probable that the new drugs of the invention will be useful in the treatment of the said diseases.

SUMMARY OF THE INVENTION

The basic aspect of the invention are new derivatives of 4,5,6,7-tetrabromobenzimidazole of formula 1



Formula 1

wherein R_1 is hydrogen or aliphatic group, R_2 is aliphatic group optionally substituted with hydrogen or substituent such as hydroxyl group or substituted amino group.

The following compounds are new derivatives of the present invention:

2-methylamino-4,5,6,7-tetrabromo-1H-benzimidazole,

2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole,

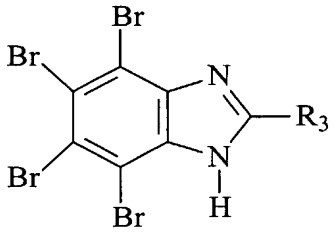
2-etanolamino-4,5,6,7-tetrabromo-1H-benzimidazole,

2-isopropyloamino-4,5,6,7-tetrabromo-1H-benzimidazole,

2-(2-hydroxypropylamino)-4,5,6,7-tetrabromo-1H-benzimidazole,

2-(2-dimethylaminoethylamino)-4,5,6,7-tetrabromo-1H-benzimidazole.

The further aspect of the invention is a method of preparation of the new derivatives of 4,5,6,7-tetrabromobenzimidazole of formula 1, wherein R_1 is hydrogen or aliphatic group, R_2 is aliphatic group optionally substituted with hydrogen or a substituent such as hydroxyl group or substituted amino group, in the reaction of a compound of formula 2,



Formula 2

wherein the substituent R_3 is halogen, alkylthio group or alkoxy group or other group that is easily substituted, with an amine, at elevated temperature, and the resulting product is subjected to purification by crystallization or chromatography on silica gel, and, then, treated with mineral acid or organic acid to convert into salt. In the compound of formula 2, the substituent R_3 is halogen such as Cl or Br, or alkylthio group ,such as CH_3S, CH_3CH_2S, $CH_3CH_2CH_2S$, or lower alkoxy group, such as CH_3O, C_2H_5O or other group easily being substituted, such as sulfone group or alkylsulfoxide group.

A lower aliphatic primary amine optionally containing in the aliphatic chain additionally hydroxyl groups or substituted amino groups is used as an amine. A lower aliphatic secondary amine can also be used as an amine in the present invention. A characteristic and advantageous feature of the method of the invention is that the amine is used both as a reagent and a solvent, in aqueous or alcoholic solution and reaction of the compound of formula 2 with amine is performed within a temperature range from 80 to 140 °C.

The resulting compounds of formula 1 can be converted by a known method into salts of mineral or organic acids.

A further aspect of the invention is a pharmaceutical composition exhibiting anti-neoplastic activity, containing effective anti-neoplastic acting amount of a new compound of the invention combined with at least one inert pharmaceutically acceptable carrier, diluent or excipient.

Compounds of the present invention can be prepared and administered in a variety of dosage forms for oral and parenteral administration. For example, compounds of the invention, can be administered through injection i.e. as intravenous, intramuscular, intradermal, subcutaneous, intraduodenal, intraperitoneal dosage form. As regards parenteral administration, liquid unit dosage forms are prepared including each of the compounds of the invention, for example 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole and aseptic vehiculum, preferably water. The above-

mentioned compounds can be suspended or dissolved in vehiculum depending on a type of pharmaceutically compatible carrier. When preparing a solution, an active ingredient can be dissolved in water for injections and sterilized by filtration. The resulting sterilized solution is filled into vials or ampoules, which are then tightly closed. After filling the vial with the preparation, it can be frozen to remove water under vacuum. The resulting dry lyophilized powder is tightly closed in the vial and the second vial is attached containing water for preparation of injection solution. The compounds can be also administered by inhalation or transdermally. It is obvious for the skilled in the art that the presented dosage forms as the active ingredient can include the new compounds as well as pharmaceutically acceptable salt of each of the compounds of the invention.

Pharmaceutically acceptable carriers, used for preparation of pharmaceutical compositions, can be liquid or solid. Solid preparation include powders, tablets, pills, capsules and dispersed granules. A solid carrier can be one or more substances, which can act as diluents, solvents, binders, preservatives, disintegrants or capsule-forming material.

The further aspect of the invention is use of the new derivatives of the invention for manufacturing of drug that has anti-neoplastic activity.

Another aspect of the invention is also a method of inhibiting caseine kinase 2 activity in patients in need of such treatment by administration of effective amount of new derivatives of the invention.

The present invention will be more readily understood on a basis of specific examples accompanied by appended figures (Fig) which are intended to illustrate the invention, and not to limit its scope.

Description of figures (Fig):

Fig.1. illustrates cytotoxic effect of 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole on human leukemia Jurkat T cells.

Fig.2. illustrates mechanism of apoptosis activity of 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole on human leukemia Jurkat cells by caspase activation..

Synthesis and characterization of tetrabromobenzimidazole (TBI) derivatives:

All chemicals and solvents used for the synthesis were purchased from Sigma-Aldrich. Melting points (uncorr.) were measured in open capillary tubes on a Gallenkamp-5 melting point apparatus. Ultraviolet absorption spectra were recorded on Kontron Uvikon 940 spectrometer. ^1H NMR spectra (in ppm) were measured with Varian Gemini 200 MHz and Varian UNITYplus 500 MHz spectrophotometers at 298 °K in D_6 (DMSO) using tetramethylsilane as the internal standard, mass spectra (70 eV) were obtained with AMD-604 (Intectra) spectrometer. Flash chromatography was

performed on silica gel (Merck) (230-400 mesh). Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel F_{254} (Merck) plates (0.25 mm thickness).

Example I. 2-Methylamino-4,5,6,7-tetrabromo-1H-benzimidazole.

The mixture of 2-chloro-4,5,6,7-tetrabromobenzimidazole (0.94 g, 2 mmol) and methylamine (EtOH sol., 35 ml, 30%) was heated 20h in steel autoclave at 110-115 °C. The reaction mixture was evaporated to dryness and the residue crystallized from 80% EtOH to give colorless crystals (630mg, 68%). M.p. 283-285 °C. TLC ($CHCl_3$/MeOH, 9:1): Rf = 0.48. ^{1}H-NMR (D_6(DMSO)): 2.94 (d, CH_3), 6.69 (q, NH), 11.60 (bs, NH-benzim.). Anal. calcd for $C_8H_5Br_4N_3$ (462.77): C, 20.76; H, 1.09; N, 9.08. Found: C, 20.70; H, 1.25; N, 8.83.

Example II. 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole.

The mixture of 2,4,5,6,7-pentabromobenzimidazole (1.02 g, 2 mmol) and dimethylamine (EtOH sol., 30 ml, 30%) was heated 20h in steel autoclave at 110-115 °C. The reaction mixture was evaporated to dryness and the residue crystallized from 80% EtOH to give colorless crystals (710 mg, 75%). M.p. > 330 °C (decomp.). TLC ($CHCl_3$/MeOH, 9:1): Rf = 0.48. ^{1}H-NMR (D_6(DMSO)): 3.13 (s, 2 × CH_3), 11.50 (bs, NH-benzim.). Anal. calcd for $C_9H_7Br_4N_3$ (476.79): C, 22.67; H, 1.48; N, 8.81. Found: C, 22.71; H, 1.61; N, 8.68.

Example III. 2-Ethanolamine-4,5,6,7-tetrabromo-1H-benzimidazole.

The mixture of 2-methylthio-4,5,6,7-tetrabromobenzimidazole (0.96 g, 2 mmol) and ethanolamine (10 ml) was heated 8h under reflux in the oil bath at 120 °C. The reaction mixture was evaporated to dryness and the residue crystallized from 80% EtOH to give colorless crystals (610 mg, 62%). M.p. 216-218 °C. TLC ($CHCl_3$/MeOH, 95:5): Rf = 0.48. ^{1}H-NMR (D_6(DMSO)): 1.05 (t, CH_2), 4.36 (t, CH_2), 4.90 (bs, OH), 6.68 (t, NH), 11.50 (bs, NH-benzim.). Anal. calcd for $C_9H_7Br_4N_3O$ (492.79): C, 21.94; H, 1.43; N, 8.53. Found: C, 21.90; H, 1.60; N, 8.40.

Example IV. 2-Isopropylamine-4,5,6,7-tetrabromo-1H-benzimidazole.

The mixture of 2,4,5,6,7-pentabromobenzimidazole (1.02 g, 2 mmol) and isopropylamine (EtOH sol., 25 ml, 20%) was heated 20h in steel autoclave at 110-115 °C. The reaction mixture was evaporated to dryness and the residue crystallized from 80% EtOH to give colorless crystals (690 mg, 70%). M.p. 288-290 °C (decomp.). TLC ($CHCl_3$/MeOH, 9:1): Rf = 0.74. ^{1}H-NMR (D_6(DMSO)): 1.22 (d, 2 × CH_3), 4.05 (m, CH), 6.60 (d, NH), 11.20 (bs, NH-benzim.). Anal. calcd for $C_{10}H_9Br_4N_3$. (490.82): C, 24.47; H, 1.85; N, 8.56. Found: C, 24.36; H, 1.98; N, 8.40.

Example V. 2-(2-hydroxypropylamino)-4,5,6,7-tetrabromo-1H-benzimidazole.

The mixture of 2,4,5,6,7-pentabromobenzimidazole (1.02 g, 2 mmol) and 2-hydroxypropylamine (H_2O sol., 25 ml, 10%) was heated 20h in steel autoclave at 110-115 °C. The reaction mixture was evaporated to dryness and the residue crystallized from 80% EtOH to give colorless crystals (620 mg, 61%). M.p. 258-260 °C . TLC ($CHCl_3$/MeOH, 9:1): Rf = 0.45. ^{1}H-NMR (D_6(DMSO)): 1.12 (d, CH_3), 3.30 (m, CH), 3.83 (m, CH), 4.96 (d, OH), 6.62 (t, NH), 11.20 (bs, NH-benzim.). Anal. calcd for $C_{10}H_9Br_4N_3O$. (506.82): C, 23.70; H, 1.79; N, 8.29. Found: C, 23.66; H, 1.88; N, 8.16.

Example VI. 2-(2-Dimethylaminoethylamino)-4,5,6,7-tetrabromo-1H-benzimidazole.

The mixture of 2,4,5,6,7-pentabromobenzimidazole (1.02 g, 2 mmol) and dimethylaminoethylamine (EtOH sol., 25 ml, 10%) was heated 20h in steel autoclave at 110-115 °C. The reaction mixture was evaporated to dryness and the residue crystallized from 80% EtOH to give colorless crystals (580 mg, 56%). M.p. > 250 °C (decomp.). TLC ($CHCl_3$/MeOH, 7:3): Rf = 0.15. ^{1}H-NMR (D_6(DMSO)): 2.29 (s, 2 × CH_3), 2.54 (m, CH_2), 3.43 (q, CH_2), 6.82 (t, NH), 11.10 (bs, NH-benzim.). Anal. calcd for $C_{11}H_{12}Br_4N_4$. (519.86): C, 25.41; H, 2.33; N, 10.78. Found: C, 25.56; H, 2.48; N, 10.64.

Example VII. 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole hydrochloride.

2-Dimethylamino-4,5,6,7-tetrabromobenzimidazole (0.48g, 1mmol) was dissolved by heating in EtOH (80 ml)and to this solution hydrochloric acid (36% H_2O sol., 5 ml) was added. Almost immediately the microcrystalline solid begins to separate. The mixture was kept in 4 °C overnight and hydrochloride (350 mg, 68%) was separated as small needles. M.p. > 330 °C (decomp.).

References:

1. Navarete-Vazquez et al. Bioorg.Med.Chem.Lett.11, (2001), 187-191;
2. Andrzejewska et al.Eur.J.Med.Chem. 37 (2002)972-978).
3. Druker B.J., Talpaz M., Resta D.J., Peng B., Buchdunger E., Ford J.M., Lydon Nn.B., Kantarjian H., Capdeville R., Ohno-Jones S., SawyersC.L. *N. Eng. J. Med.* 344, (2001) 1031-1037.
4. Litchfield D.W. *Biochem. J.* 369, (2003) 1-15.
5. Kelliher M.A., Seldin D.C., Leder P. *EMBO J.* 15, (1996) 5160-5166.
6. Orlandini M., Semplici F., Feruzzi R., Meggio F., Pinna L.A., Oliviero S. *J. Biol. Chem.* 273, (1998) 21291-21297.
7. Landesman-Bollag E., Channavajhala P.L., Cardiff R.D., Seldin D.C. *Oncogene* 16 (1998) 2965-2974.